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# **Discriminating selection on lateral plate phenotype and its underlying gene, Ectodysplasin, in threespine stickleback**

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## Abstract

When a signature of natural selection is discovered on a gene that is pleiotropic or in tight linkage with other genes, it is challenging to determine which of the affected phenotypes is under selection. One way to make progress is to employ methods for analyzing natural selection on correlated traits, including both genotype and phenotype. We used this approach in threespine stickleback to estimate selection on a rapidly evolving trait, lateral armor plates, while controlling for variation at its major underlying gene, *Ectodysplasin (Eda)*, and vice versa. This allowed for independent estimates of selection on lateral plates and on *Eda* via other traits. Previously, we demonstrated allele frequency changes at *Eda* in a pond experiment. Here we show that this resulted from selection on both plates and on *Eda*, implying additional selection on other phenotypic traits affected by the same gene. This represents the first evidence for direct selection on lateral plates independent of selection on the *Eda* locus and highlights the value of measuring selection on both phenotypes and genotypes in studies of adaptation.

## Introduction

The study of adaptation seeks to establish a link between phenotypic variants, their underlying genotypes, and their fitness in a given environment. Recent advances in sequencing and genomics have enabled researchers to identify genes and genomic regions under natural selection using genome scans (e.g. Beaumont and Balding, 2004; Linnen et al., 2009; Jones et al., 2012; Therkildsen et al., 2013) and experimental studies of changes in allele frequency over time (e.g. Korves et al., 2007; Barrett et al., 2008; Burke et al., 2010; Fournier-Level et al., 2011; Pespeni et al., 2013; Gompert et al., 2014). However, pleiotropy and genetic linkage complicate the effort to identify phenotypic targets of selection. Even if a focal phenotype has been identified, a signature of selection at a locus may result instead via other traits determined by the same gene or linked genes, dragging the focal trait along as a correlated response. The challenge is similar to that faced by researchers attempting to identify which trait or traits, among a correlated suite, are the direct targets of phenotypic natural selection (Lande and Arnold, 1983; Price and Langen, 1992). Statistical methods for estimating selection on correlated phenotypic traits (Lande and Arnold, 1983) have been useful in identifying the phenotypes that are direct targets of natural selection (e.g. Grant, 1985; Schluter and Smith, 1986; Price and Langen, 1992; Nagy, 1997; Reznick et al., 1997). Here we show that the same approach can be utilized to help determine whether selection on a focal phenotypic trait has contributed to changes in genotype frequency at an underlying gene.

We apply the Lande-Arnold approach to a study of multivariate selection on armor plates and its major underlying gene, *Ectodysplasin (Eda)* (Colosimo et al., 2005), in threespine stickleback (*Gasterosteus aculeatus*). Freshwater populations established following colonization by the marine threespine stickleback after the last ice age (10-12,000 years ago) have repeatedly

evolved a reduction of bony lateral armor plates (Bell and Foster, 1994). Adult marine stickleback generally possess 30 to 36 lateral plates on each side of the body, whereas most freshwater populations founded by marine colonizers have between 0 and 9 plates (Bell and Foster, 1994). The rate of evolution of plate loss in freshwater can be rapid (Klepaker, 1993; Kristjánsson et al., 2002; Bell et al., 2004; Kristjánsson, 2005). In every known case, loss of plates in freshwater has taken place via the replacement of the “complete” armor allele at the *Eda* locus (hereafter, the C allele) by a relatively ancient “low” armor allele (the L allele) (Colosimo et al., 2005). The rapid and parallel substitution of one allele by another strongly suggests that natural selection is responsible (Simpson, 1953; Schluter and Nagel, 1995), but it does not identify the mechanism of selection. In particular, we remain uncertain whether armor plating is itself the target of selection, or whether its rapid evolution is a by-product of selection on other traits affected by the same underlying gene.

Number of lateral plates has long been regarded as the direct target of natural selection (Bell and Foster, 1994), and there is some evidence in support of this hypothesis (Hagen and Gilbertson, 1973; Bell et al., 2004; Raeymaekers et al., 2007; Kitano et al., 2008; Leinonen et al., 2011; DeFaveri and Merilä, 2013). However, no study has distinguished selection on lateral plates from selection on other traits that may be controlled by *Eda* or other tightly linked genes. In a previous study we detected strong selection at the *Eda* locus in marine stickleback transplanted to freshwater ponds (Barrett et al., 2008). The experiment was conducted to test the “growth hypothesis” for the adaptive value of low armor plating in freshwater, namely, that low armor is favored because of the high cost of mineralizing bone under reduced ion availability (Giles, 1983; Bell et al., 1993; Marchinko and Schluter, 2007). The experiment tested the prediction that low-armor phenotypes would have faster growth, leading to higher survival and

earlier reproduction, by measuring relative fitness of genotypes at the underlying *Eda* gene. All transplanted adult marine stickleback were heterozygous at the *Eda* locus (CL genotype), and they bred in artificial ponds to produce a cohort of all three genotypes (CC, CL, and LL) whose frequencies were then tracked over the course of a year. Some of the results of the study supported the growth hypothesis. In particular, mean body length was positively correlated with the number of low alleles a fish possessed. Barrett and colleagues (2008) also found strong selection at the *Eda* locus. No such selection on *Eda* was detected in similar crosses raised in the lab (Barrett and Schluter, 2010). These results are consistent with the growth hypothesis; however, the study did not directly measure selection on lateral plates. Thus, it remains to be determined whether the observed changes in *Eda* genotype frequencies were the result of selection on plates or selection on other (unknown) traits affected by the *Eda* locus.

Here we estimate the strength of selection on lateral plates while controlling for *Eda* genotype. This is possible because genetic and phenotypic variation in lateral plate number is present within *Eda* genotypes (Colosimo et al., 2005). If selection acted on lateral plates, it should be detectable even while holding *Eda* genotype constant. Conversely, if genotype frequency changes at *Eda* resulted only via its pleiotropic effects on other selected traits, then holding *Eda* genotype constant should eliminate apparent selection on plates. In this case, holding plate number constant should have little effect on the estimated strength of selection on *Eda* genotype.

## Materials and Methods

### *Pond Experiment*

The original experiment is fully described in Barrett et al. (2008). Briefly, 45 or 46 wild adult marine threespine stickleback heterozygous at the *Eda* locus were introduced into four experimental freshwater ponds on the University of British Columbia campus. The fish were allowed to reproduce and, beginning in August 2006, 50 F<sub>1</sub> progeny per pond per month were destructively sampled, assayed for standard length, and genotyped at a diagnostic marker for the *Eda* locus (Colosimo et al., 2005). The allele and genotype frequencies of the four F<sub>1</sub> populations were then compared over the course of a year.

Here we focus on the three samples of juvenile fish taken in September, October, and November 2006, during which the strongest changes in genotypes frequencies were detected at the *Eda* locus (Fig. 1). Selection increased the CC genotype frequency by 19% between September and October, and lowered the CL genotype frequency an equal amount. C allele frequency decreased from October to November, however, driven by a 15% decrease in CC genotype frequency and a 12% increase in CL genotype frequency. During both periods the LL genotype frequency remained largely unchanged.

### *Lateral Plate Phenotype*

We measured the number of lateral plates in a random sample of individuals from each of the three genotypes, yielding a total of between 76 and 85 fish from each of the September, October, and November samples in the Barrett et al. (2008) experiment (241 fish in total). Fish specimens were fixed in 10% formalin and stained with 0.001-0.002% w/v alizarin red S powder



in a 2% w/v potassium hydroxide solution. Stained fish were then photographed under standardized conditions and the total number of lateral plates on the left side was counted.

Lateral plate number was positively correlated with standard length, a measure of fish body size, particularly in CC and CL genotypes (CC,  $r = 0.83$ ,  $P = 2.07 \times 10^{-13}$ ; CL,  $r = 0.53$ ,  $P = 4.94 \times 10^{-12}$ ; LL,  $r = 0.07$ ,  $P = 0.45$ ), indicating that the number of plates had not completed development in many of the juvenile individuals in the samples. We used a breakpoint regression method (Fig. A1) to size-adjust plate number, as follows: we fit a model in which the logarithm of plate number  $y$  increased linearly with fish length  $x$  up to a threshold value,  $x^*$ , beyond which no further change took place:

$$Y = a + bx \quad \text{for } x < x^*$$

$$Y = a + bx^* \quad \text{for } x \geq x^*$$

The constants  $a$ ,  $b$ , and  $x^*$  were estimated from the data using nonlinear least squares (using the “nls” function in R, version 2.15.0; R Development Core Team 2012). Log plate counts were used in this analysis to reduce the skew of the data. The intercept and slope,  $a$  and  $b$ , were free to vary between *Eda* genotypes. We assumed that the threshold size  $x^*$  was the same for all three *Eda* genotypes, since more complex models that also varied the threshold  $x^*$  between genotypes did not improve the fit, as judged by AIC scores. Incorporating separate coefficients for each of the four ponds also did not improve the fit, and the analyses we present here do not include pond effects. The threshold  $x^*$  value was estimated to be  $34.0 \text{ mm} \pm 1.3 \text{ SE}$  (Fig. A1). In our selection analyses, log plate number for each fish was size-adjusted to a body length of 34.0 mm. Adjusted log plate counts were then back-transformed to the original and more intuitive non-log scale. Adjusted plate counts exceeding the value 32 were reduced to 32 to ensure that the range did not exceed the natural maximum seen in our data set.

### *Selection Analysis*

We estimated selection coefficients (standardized partial regression coefficients) using the method for cross-sectional data (Lande and Arnold, 1983):

$$\hat{\boldsymbol{\beta}} = \mathbf{P}^{-1}[\bar{\mathbf{x}}_{\text{after}} - \bar{\mathbf{x}}_{\text{before}}],$$

where  $\hat{\boldsymbol{\beta}}$  is the vector of estimated selection coefficients,  $\bar{\mathbf{x}}$  is the vector of means of the focal phenotype trait and genotype scores (hereafter, “traits”) before and after selection, and  $\mathbf{P}$  is the matrix of variances and covariances of the traits before selection. Separate analyses were carried out for the two episodes of selection, one between September and October 2006, and the other between October and November 2006 (Fig. 1).

The vectors of trait means included the size-adjusted number of plates as the focal phenotypic trait. Genotype was scored using two genotype indicator variables. The first genotype variable (“additive”) coded the LL genotype as -1, the CL genotype as 0, and the CC genotype as 1. The second genotype variable (“dominance”) coded homozygous genotypes (LL and CC) as 0 and the heterozygotes (CL) as 1. All selection coefficients were standardized by multiplying each partial regression coefficient by the standard deviation of the trait before selection, to allow comparison between trait and genotype scores measured on different scales (Lande and Arnold 1983). Since *Eda* genotype is categorical and lateral plate number is numerical, the method used here is an analysis of covariance to tease apart the contributions of genotype and plates to relative survival. Note that  $\hat{\boldsymbol{\beta}}$  is not strictly a selection gradient because the traits do not have a multivariate normal distribution.

Our analysis is within a single generation, and hence requires no assumptions about the probability distribution of breeding values of the numeric trait. Lande (1983) presents the theory

for predicting evolutionary response to selection on a quantitative trait influenced by a major locus and having an otherwise additive genetic basis. We do not predict evolutionary response here because our focus is on selection.

We also calculated standardized univariate selection intensities ( $s'$ ) separately for lateral plates and the genotype variables as:

$$s' = (\bar{x}_{\text{after}} - \bar{x}_{\text{before}}) / \hat{\sigma}_{\text{before}},$$

where  $\bar{x}_{\text{before}}$  and  $\bar{x}_{\text{after}}$  are the phenotype or genotype trait means (coded in the same way as the dominance variable) before and after selection and  $\hat{\sigma}_{\text{before}}$  is the estimated standard deviation of the trait before selection.

For simplicity, our analysis used size-adjusted lateral plates. However, we carried out an additional analysis in which size (standard length) was included as a trait along with unadjusted lateral plate number and *Eda* genotype. To accommodate the nonlinear relationship between plate number and size, it was necessary to adjust the standard length of the largest individuals to a maximum of 34.0 mm, the estimated breakpoint (Fig. A1). The results of this analysis were not quantitatively different from the simpler analysis using size-adjusted plates and we do not present it.

To simplify further, our first analysis excluded the LL genotype and retained the CL and CC genotypes (Table 1). In this case there is only one genotype variable in the linear model (CL genotype is scored as 1 and CC is scored as 0), which incorporates the dominance component of *Eda* genotype but also half the additive component. This simplification is justified because CC and CL genotypes have high variance (Table A2) and overlap in plate number, which is required to disentangle the separate effects of plates and genotypes on fitness. The variance-covariance matrices for the one-genotype analysis are given in Tables A3 and A4 (one for each pair of

months). In contrast, there is little plate number variation within the LL genotype, and little overlap in plate number with the other genotypes. A second analysis that included the LL genotype and the dominance variable is reported in the online appendix (Table A1; see Tables A5 and A6 for the variance-covariance matrices).

We generated 95% confidence intervals for partial regression coefficients for each pair of months using a bootstrap resampling procedure. Each bootstrap replicate involved resampling with replacement, for a given month, the genotypes of  $n$  individuals from the corresponding Barrett *et al* (2008) data set, where  $n$  is the number of individuals measured in our sample (the number for which we have lateral plate measurements). Next, for each genotype  $i$  we resampled  $n_i$  phenotypes from the distribution of lateral plates corresponding to each genotype in the data. Resampling was done 10,000 times. Standardized partial selection coefficients were then calculated on each bootstrap replicate. The 0.025 and 0.975 quantiles of the coefficients were used to calculate the 95% confidence intervals of the parameters. The 95% confidence intervals of the selection intensities were estimated in the same way.

All our analyses incorporate pond as a fixed effect, essentially treating individual fish as the unit of replication. This is in contrast to the Barrett *et al.* (2008) study, which treated pond as a random effect and the unit of replication. Our approach here is justified because our goal is not to test for selection generally, as in the case of Barrett *et al.* (2008), but rather to investigate more narrowly the targets of selection within an experiment that has already demonstrated selection. All of the ponds responded similarly, and including pond in the analysis did not affect the results. For simplicity we present only the results for the analyses without the pond variables.

All statistical analyses were conducted in R (version 2.15.0, R Development Core Team 2012).

## Results

We detected strong selection both on *Eda* genotype and on lateral plates (Table 1; Table A1) using multivariate methods. Between September and October, selection favored the *Eda* CC genotype over the CL genotype: point estimates of selection for the genotype variable and phenotypic trait, lateral plates, were similar in magnitude (Table 1), although the 95% confidence interval for lateral plates narrowly spanned zero. Between October and November the direction of selection changed, now favoring the CL genotype. During this period, selection on lateral plates and genotype was slightly weakened and the confidence intervals for both variables spanned zero (Tables 1). Thus, selection on plates and genotype could not be disentangled in this episode. These analyses include only two genotypes and hence a single genotype variable that lumped additive and dominance components. When we analyzed all three genotypes in the September-October episode by including two genotype variables, one for each of the additive and dominance components, we detected strong selection on the dominance component of genotype (Table A1). The CL genotype was disfavored, in agreement with the findings of Barrett et al., (2008), who detected heterozygote underdominance for fitness. The point estimate of selection on plates was positive, but the confidence interval (barely) spanned zero. All confidence intervals for selection between October and November spanned zero, again indicating that selection on plates and genotype could not be disentangled in this episode (Table A1).

If we had limited our analyses to univariate estimates of selection ( $s'$ ), which by definition do not account for selection on correlated characters, the picture would have appeared different. The mean number of adjusted plates changed only slightly between September (18.7  $\pm$  0.91 SE), October (19.01  $\pm$  1.04 SE) and November (18.65  $\pm$  0.89 SE) (Fig. A2).

Consequently, the estimates of selection intensity indicated much weaker selection on lateral plates relative to selection on *Eda* genotype, which was at least an order of magnitude greater (Table 2). Additionally, unlike the selection intensity estimates for *Eda* genotype, the selection intensity estimates for lateral plates were near zero. Correspondingly, if selection estimates on lateral plates were only univariate (Table 2) the selection on lateral plates that was detected in the multivariate analysis (Table 1) would have been missed.

## Discussion

We compared the magnitude of selection on a trait and its major underlying gene to investigate the targets of selection during two episodes. Univariate estimates of selection indicated strong selection on *Eda* genotype and only weak selection on lateral plate phenotype (Table 2). Our aim was to determine whether changes in *Eda* genotype frequency within a cohort in an experiment were due to selection on lateral plates themselves, as has been repeatedly suggested (Hagen and Gilbertson, 1973; Reimchen, 1992; Bell et al., 2004; Kitano et al., 2008), or the result of selection on some other unmeasured traits affected by the same underlying gene. Using Lande-Arnold (1983) methods for correlated characters, we found that selection on lateral plates was of similar strength as selection on *Eda* genotype in one of two episodes (Tables 1 and A1), although barely non-significant. In the second episode, confidence limits for both selection coefficients spanned zero and thus selection on plates and genotype could not be disentangled (Tables 1 and A1). The three-genotype analysis incorporating a dominance variable (Table A1) indicates that the selection on *Eda* genotype (Table 1) was largely the result of selection against heterozygotes, rather than on the additive component of genotype; this heterozygote underdominance for fitness was previously suggested by Barrett et al. (2008).

These results suggest that the rapid changes in genotype frequency at the *Eda* locus during experimental freshwater introduction were partially due to selection on lateral plate phenotype and partially due to selection on additional, unmeasured traits controlled by *Eda* or a tightly linked gene. However, genotype and phenotype are strongly correlated ( $r = 0.698$ ), and their separate effects on fitness could not be separated in the second episode (October - November). While the analysis of selection on correlated traits has its limitations (discussed by Mitchell-Olds and Shaw, 1987), we suggest these methods may help to disentangle whether selection is directly or indirectly influencing a phenotypic trait of interest when genotype at its major underlying locus is known.

Direct selection on lateral plate phenotype has long been thought to be the main factor driving the repeated evolution of reduced lateral plate armor in freshwater populations (Hagen and Gilbertson, 1973; Reimchen, 1992; Bell et al., 2004; Kitano et al., 2008; Leinonen et al., 2011). A number of studies have suggested that plate reduction has been favored in freshwater due to an increased cost of mineralizing bone in freshwater due to reduced ion availability relative to marine environments (Giles, 1983; Bell et al., 1993). This hypothesis has some empirical support; Marchinko and Schluter (2007) and Barrett et al. (2008) found that low-plated genotypes grew faster in fresh water. Other work suggests that reduced predation in freshwater environments relative to the marine environment is responsible for lateral plate reduction (Moodie et al., 1973; Reimchen, 1992; Reimchen, 2000). Lateral plate reduction has also been suggested to improve swimming ability by increasing burst speed and buoyancy, which may aid in predation avoidance (Bergstrom, 2002; Myhre and Klepacker, 2009). However, until now no study had disentangled the direct effect of plates on growth, and survival from the effects of *Eda* via other unmeasured phenotypic traits.

Two previous studies of geographic variation in lateral plates and *Eda* genotype frequencies found that genetic variance among populations ( $F_{st}$ ) at *Eda* was lower than phenotypic variance in lateral plates ( $Q_{st}/P_{st}$ ) (Raeymaekers et al., 2007; DaFaveri and Merilä, 2013), relative to total variance. This suggested stronger divergent selection among populations on lateral plates than on *Eda* frequencies. These results are consistent with our findings of selection on plates in a pond experiment. However, when we estimated the direct contribution of lateral plates to changes in *Eda* genotype frequencies, we found that lateral plates only partially explain these changes. Statistically controlling for number of lateral plates did not eliminate the signal of strong selection on *Eda*. These results highlight the value of measuring selection on a trait and its major underlying gene to help identify targets of selection.

On the basis of these results, we suggest that the rapid and repeated evolution of low-plated armor in freshwater may be the result of both selection on lateral plates and a correlated response to selection on other unmeasured traits affected by *Eda*. *Eda* has been suggested to have diverse pleiotropic effects (Barrett et al., 2009a; Saider et al., 2013). For example *Eda* has been shown to affect the number of neuromasts along the lateral line (Wark et al., 2012; Mills et al., 2014). It is currently unclear if *Eda*'s effect on neuromast distribution is direct or whether it is mediated indirectly through lateral plate development (Mills et al., 2014). Thus, there is potential that some of the selection that we detect at the *Eda* locus and/or lateral plates is due to selection on the lateral line sensory system. In addition to its role in lateral plate and neuromast development, variation at *Eda* is associated with variation in schooling behavior (Greenwood et al., 2013) and propensity to switch between water conditions of varying salinity (Barrett et al. 2009b). In addition, selection might be acting on traits controlled by genes in linkage



disequilibrium with *Eda* (Colosimo et al., 2005), which lies in a region of low recombination (Hohenlohe et al., 2011).

This study serves as a reminder that although genetic and genomic studies are informative about the evolution of traits, alone they provide insufficient evidence for selection on those traits, even when the link between a particular genotype and phenotype appears clear. Correspondingly, estimates of natural selection on phenotypes will remain an important component in genomic studies of adaptation (Travisano and Shaw, 2013) and are required to indicate whether mechanisms such as correlated selection are at work.

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## Online Appendix: Supporting Information

**Table A1:** Standardized partial selection coefficients for the lateral plate phenotype, *Eda* genotype, and dominance for the September to October and October to November periods. 95% confidence intervals are reported in brackets.

	September-October $\hat{\beta}$	October-November $\hat{\beta}$
Lateral plates	0.35 (-0.08, 0.78)	-0.16 (-0.83, 0.40)
Additive genotype	-0.04 (-0.43, 0.38)	-0.04 (-0.65, 0.69)
Dominance	-0.47 (-0.83, -0.14)	0.26 (-0.14, 0.71)

**Table A2:** Variance in lateral plate number for each *Eda* genotype by month.

	September	October	November
CC	22.1	25.8	24.3
CL	19.7	19.1	18.7
LL	9.5	8.4	8.7

**Table A3:** Variance covariance matrix for the standardized traits included in the one-genotype variable Lande-Arnold analysis for the September – October period.

	Lateral Plates	Additive Genotype
Lateral plates	0.70	0.17
Genotype	0.17	0.41

**Table A4:** Variance covariance matrix for the standardized traits included in the one-genotype variable Lande-Arnold analysis for the October – November period.

	<b>Lateral Plates</b>	<b>Additive Genotype</b>
Lateral plates	0.63	0.23
Genotype	0.23	0.40

**Table A5:** Variance covariance matrix for the standardized traits included in the two-genotype variable Lande-Arnold analysis for the September – October period.

	<b>Lateral Plates</b>	<b>Additive Genotype</b>	<b>Dominance</b>
Lateral plates	1.04	0.68	0.06
Additive genotype	0.68	0.10	-0.16
Dominance	0.06	-0.16	1.01

**Table A6:** Variance covariance matrix for the standardized traits included in the two-genotype variable Lande-Arnold analysis for the October – November period.

	<b>Lateral Plates</b>	<b>Additive Genotype</b>	<b>Dominance</b>
Lateral plates	1.05	0.81	0.01
Additive genotype	0.81	1.07	-0.14
Dominance	0.01	-0.14	0.94

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## Tables

**Table 1:** Standardized partial selection coefficients for lateral plate phenotype and *Eda* genotype for the September to October, and October to November, episodes. 95% confidence intervals are reported in parentheses.

	September-October $\hat{\beta}$	October-November $\hat{\beta}$
Lateral plates	0.34 (-0.04, 0.74)	-0.21 (-0.66, 0.22)
<i>Eda</i> genotype	-0.42 (-0.07, -0.79)	0.26 (0.77, -0.23)

**Table 2:** Standardized univariate selection intensities for *Eda* genotype and lateral plate phenotype. 95% confidence intervals are reported in parentheses.

	September-October $s'$	October-November $s'$
Lateral plates	0.04 (-0.29, 0.38)	-0.04 (-0.33, 0.25)
<i>Eda</i> genotype	-0.39 (-0.21, -0.56)	0.28 (0.07, 0.52)

## Figure Legends

**Figure 1:** *Eda* genotype frequencies of marine stickleback after introduction to freshwater ponds. CC: carries two copies of the “complete” armor allele; CL: carries one “complete” allele and one “low” allele; LL: carries two copies of the “low” allele. Data points represent the mean of four ponds; error bars represent the standard error of the mean. Data re-plotted with permission from Barrett et al. (2008).

**Online Figure A1:** Relationship between length and number of lateral plates. Fish reach their adult number of lateral plates at ~34 mm in length, indicated by the broken line. Solid lines indicate the fitted breakpoint regression, Black: CC genotype fish; light grey: CL genotype fish; dark grey: LL genotype fish.

**Online Figure A2:** Change in size-corrected lateral plate number for all genotypes across the sampling period. The horizontal black line indicates the sample median and the boxes denote the interquartile range.

Figure 1

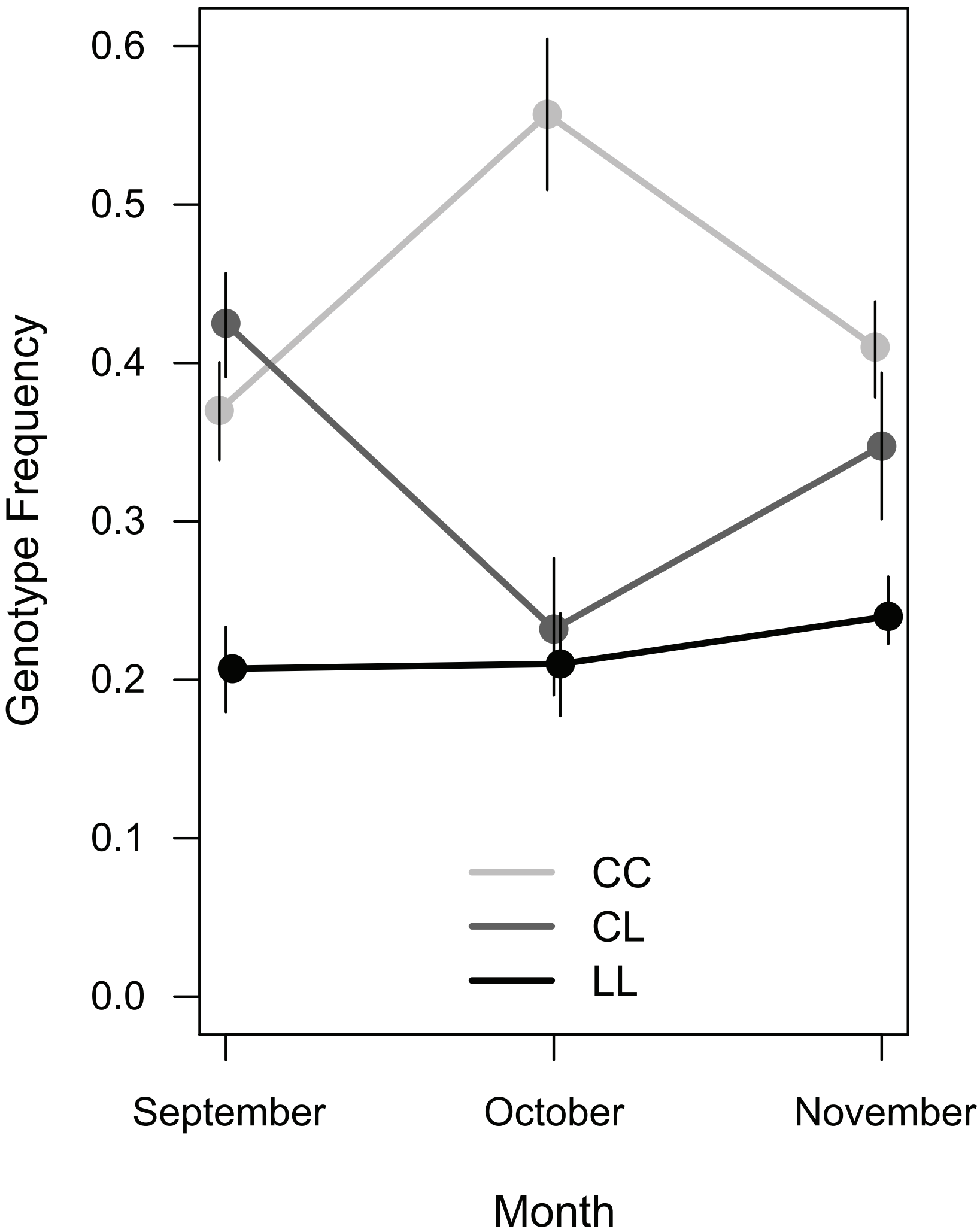


Figure A1

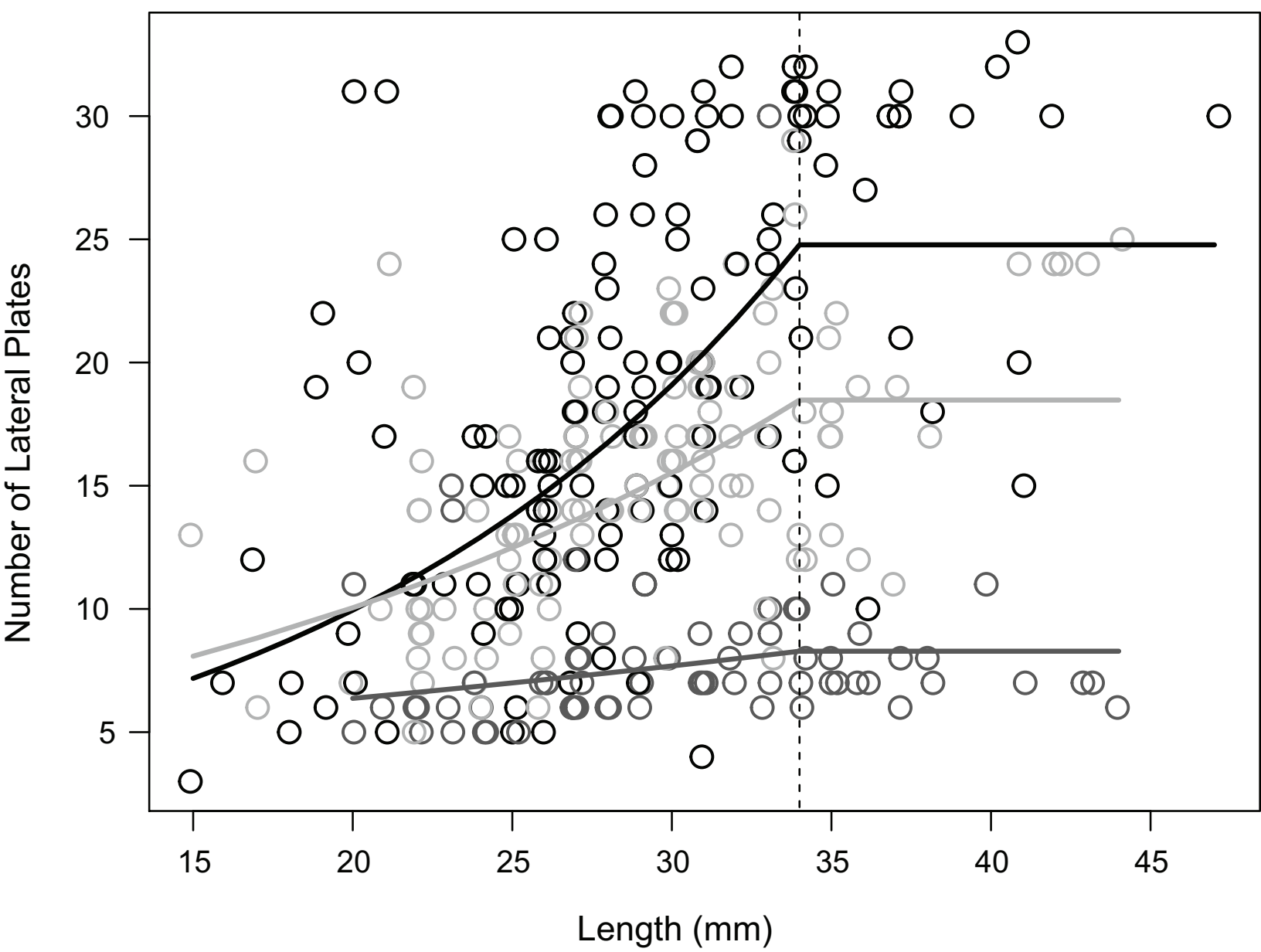


Figure A2

